Invasive Disease Caused Simultaneously by Dual Serotypes of *Streptococcus pneumoniae*

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ABSTRACT There are at least 98 known pneumococcal serotypes. Invasive pneumococcal disease (IPD) is usually caused by a single serotype, and dual-serotype IPD is rare. To assess factors associated with dual-serotype IPD, patient information obtained through laboratory-based surveillance for IPD from 2005 through 2014 in South Africa was reviewed. Genomes of isolate pairs from coinfecting individuals were sequenced to determine their molecular characteristics. For 30 (91%) of 33 patients with dual serotypes, one or both isolates were a pneumococcal conjugate vaccine (PCV13) serotype. Dual-serotype IPD was associated with children <5 years of age (adjusted odds ratio [aOR], 4.7; 95% confidence interval [95% CI], 1.8 to 11.7), underlying illness (other than HIV) (aOR, 2.8; 95% CI, 1.1 to 6.6) and death (aOR, 2.5; 95% CI, 1.08 to 6.09). For each coinfecting pair, isolates were genotypically unrelated, and their genotypes were common among isolates of the same serotype in South Africa. Of 701 accessory genes identified among dual-serotype IPD isolates, four were common between isolate pairs. Coinfecting isolate pairs had different genotypic backgrounds. The association of dual serotypes with death warrants increased awareness of IPD coinfection caused by two or more serotypes.

KEYWORDS dual, invasive, pneumococcal, serotype

Streptococcus pneumoniae (pneumococcus) is commonly carried asymptomatically in the human nasopharynx. Occasionally, it evades the immune system, crosses the mucous membranes, and is an important cause of invasive disease such as meningitis and bacteremia and noninvasive disease such as acute otitis media (1). Currently, there are at least 98 serotypes of the pneumococcus that differ in their abilities to cause invasive pneumococcal disease (IPD) (2). Since the pneumococcus is naturally competent, colonization provides an opportunity for genetic recombination between cocolonizing pneumococci or with other closely related species. This may result in phenotypic changes such as antibiotic resistance or capsular switching (3).

Individuals may be carriers of more than one serotype (4); however, IPD caused by more than one serotype simultaneously in an individual is rarely reported (5–7). The Quellung reaction remains the gold standard method for pneumococcal serotyping but is reliant on bacterial isolation and requires serotyping of multiple colonies to detect multiple serotypes in a specimen. Molecular techniques, such as real-time PCR and microarray, determine serotype directly from a clinical specimen and therefore have increased sensitivity to detect multiple serotypes present in a specimen (8–10).

Apart from Hiller et al. (3), who analyzed six isolates belonging to two distinct

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pneumococcal strains obtained over a period of 7 months from a patient with chronic pneumococcal infection, we are aware of only two other studies that characterized pneumococcal isolates obtained simultaneously from the same disease episode (5, 6). Using pulsed-field gel electrophoresis, both studies showed that the isolates had different serotypes and genotypes. Whole-genome sequencing (WGS) and the continuous development of bioinformatics software for analysis of such data enable an in-depth analysis of genomes and the detection of possible interaction (such as horizontal recombination) between isolates.

The 7-valent pneumococcal conjugate vaccine (PCV7) has routinely been used in South Africa since 2009, administered in a three-dose schedule at 6, 14, and 36 weeks of age, and was replaced with a 13-valent vaccine (PCV13) in 2011.

Through laboratory-based surveillance for IPD in South Africa from 2005 to 2014, we identified individuals coinfecting with more than one pneumococcal serotype. In this study, we describe characteristics of these patients and identify factors associated with dual-serotype IPD. We sequenced genomes of isolate pairs collected from these individuals to determine their molecular characteristics between coinfecting isolates.

MATERIALS AND METHODS

National surveillance. Laboratory-based surveillance for IPD was initiated in 1999 (11). For the present study, we reviewed cases and used isolates collected from 2005 through 2014. Patients with IPD were defined as individuals from whom *S. pneumoniae* was isolated from specimens that are normally sterile (e.g., cerebrospinal fluid [CSF], blood, or joint fluid) (12). Primary testing and culture were conducted at clinical diagnostic laboratories. Isolates and patient data were submitted to the National Institute for Communicable Diseases (NICD) from approximately 200 participating laboratories from all nine provinces. Since 2003, additional patient information, including HIV status and outcome, were collected at 24 enhanced surveillance sentinel sites located in the nine provinces. Isolates were received on Dorset transport media (Diagnostic Media Products [DMP], Johannesburg, South Africa) and subcultured on arrival on 5% horse blood agar plates (DMP) and demonstrated optochin susceptibility (Mast Diagnostics, Virginia, USA) (13). Cultures were cryopreserved in 10% skim milk (DMP) at -70°C .

Serotyping and antimicrobial susceptibility testing. Serotype was determined by the Quellung method using serotype-specific antisera (Statens Serum Institute, Copenhagen, Denmark) (14). All isolates were initially screened for antimicrobial susceptibility by disc diffusion and MIC were determined by agar dilution or Etest (AB Biodisk, Solna, Sweden) from 2005 through 2008 and, from 2009 onwards, by the broth microdilution method using commercially prepared Sensititre-SASP2 panels (Trek Diagnostics, Inc., Cleveland, OH). Results were interpreted according to the Clinical and Laboratory Standards Institute guidelines and breakpoints (15). The following antimicrobial agents were tested: penicillin G, ceftriaxone, amoxicillin, erythromycin, clindamycin, chloramphenicol, tetracycline, rifampin, cotrimoxazole, ofloxacin, linezolid, and vancomycin. Isolates were considered nonsusceptible to penicillin at MICs ≥ 0.12 mg/liter using the oral penicillin meningitis breakpoints. For other antimicrobials, isolates were defined as nonsusceptible if they were intermediately or fully resistant to the agent tested. Isolates were considered to be multidrug resistant if they were nonsusceptible to at least three classes of antimicrobials.

Identification of patients coinfecting with more than one serotype (dual-serotype IPD). Serotyping was performed on a sweep of colonies. However, if different colony morphologies were observed or a double zone of inhibition was evident around the optochin disc or during antimicrobial susceptibility screening, cultures were plated out for single colonies to tease out the different phenotypes. In addition, during serotyping, a mixture of serotypes was suspected if a culture reacted partially with a specific serum pool. In such cases, all pooled sera were tested and if a positive reaction with another pool was observed, the culture and original Dorset transport medium were retested by a different laboratory technician. Submitting laboratories were asked to send original clinical specimens and the resulting cultures, if still available, to confirm dual-serotype cases and to rule out laboratory error. In addition, we investigated the serotypes of other isolates submitted from the same laboratory at the same time, namely, whether they were the same serotypes as those detected in the mixed sample. If these procedures could not be fully documented or recreated, or contamination was suspected, all isolates for a particular case were discarded.

Epidemiological data analysis. Univariate and multivariable analyses were performed with Stata version 14 (StataCorp, Ltd., College Station, TX). We assessed factors associated with dual-serotype infection among patients with IPD from enhanced sites, where additional information such as HIV status was available, using unconditional logistic regression. Factors significant at a P of <0.1 on univariate analysis were evaluated for model inclusion and nonsignificant factors at $P > 0.05$ were dropped from the multivariable model using stepwise forward selection. All two-way interactions in the final multivariable additive model were evaluated. Two-sided P values of <0.05 were considered significant throughout.

Genome sequencing and analysis. Overnight cultures were inoculated into brain heart infusion broth (DMP) and incubated overnight at 37°C in 5% CO_2 . Bacterial prelysis and DNA extraction were performed as previously described (12). Multiplexed paired-end libraries (2×300 bp) were prepared

using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Genome sequencing was carried out on an Illumina MiSeq platform.

The paired-end reads were checked for quality, trimmed, and *de novo* assembled using the CLC Genomics Workbench version 8 (CLC, Inc., Aarhus, Denmark). The resultant contiguous sequences were ordered using Mauve (16) and *S. pneumoniae* ATCC 700669 as a reference (GenBank accession no. [FM211187](#)) and annotated using Prokka version 1.11 (17). Sequences of the seven multilocus sequence typing (MLST) genes were extracted from the assembled genomes and allele numbers and sequence types (ST) were assigned using the Bio-MLST-Check module (<http://search.cpan.org/dist/Bio-MLST-Check/lib/Bio/MLST/Check.pm>). The eBURST v3 algorithm available on the MLST website was used to determine relationships between STs (18). The minimum number of identical loci was set at 0 for group definition. The default bootstrap (1,000) was used to assign the group founder ST. A clonal complex was defined as a group of related ST's sharing six of seven identical alleles with another ST in the group. Clonal complexes were named after the group founder ST as predicted by eBURST.

Using phylogenetic similarity analysis, we compared genomes of paired isolates to at least two selected genomes of invasive pneumococcal isolates from South Africa with corresponding serotype and sequence type, collected during the same period (2005 to 2014). Analysis was based on the core genome and was performed using rapid large-scale prokaryote pan-genome analysis (roary) (19) and maximum-likelihood trees were generated using RaxML version 8 (20). Furthermore, for each isolate, roary (19) was performed using these IPD isolate sequences (of the same serotype and sequence type [PubMLST database]) to identify core and accessory genes. Core genomes consisted of genes that were present as single copies in at least 90% of isolates and, in addition, had the same sequence length in every isolate.

Transpeptidase (TPD) domain amino acid sequences of penicillin-binding protein 1a (PBP1a), PBP2b, and PBP2x were extracted from sequences using the Centers for Disease Control and Prevention's (CDC) pneumococcal typing pipeline, and PBP types were assigned as previously described (21, 22). Briefly, PBP1a, PBP2b, and PBP2x TPD amino acid sequences were extracted and compared to sequences in the CDC TPD amino acid sequence databases to assign identifiers. For each isolate, the three-number combination of the three sequences was defined as its PBP type.

Ethics and informed consent. Ethical approval for national surveillance (including consent for patient participation and collection of clinical data) and for molecular characterization was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg, South Africa (protocols M081117 and M111008, respectively).

Accession number(s). The assembled genomes of isolates have been deposited in the NCBI database under the following accession numbers: [MDYG000000000](#), [MDYE000000000](#), [MDYF000000000](#), [MDYD000000000](#), [MDYC000000000](#), [MDYB000000000](#), [MDYA000000000](#), [MDXZ000000000](#), [MDXX000000000](#), [MDXV000000000](#), [MDXW000000000](#), [MDXU000000000](#), [MDXT000000000](#), [MDXS000000000](#), [MDXQ000000000](#), [MDXR000000000](#), [MDXP000000000](#), [MDXO000000000](#), [MDXN000000000](#), [MDYH000000000](#), [MDXL000000000](#), [MDXM000000000](#), [LWGT000000000](#), [MCIW000000000](#), [LZNU000000000](#), [LZNV000000000](#), [LZNW000000000](#), [MCIX000000000](#), [LYQG000000000](#), [LYQH000000000](#), [LWGU000000000](#), [MCIY000000000](#), [MCIZ000000000](#), [NNBX000000000](#), [MDXY000000000](#), [NNBW000000000](#), [MCJA000000000](#), [NOIL000000000](#), [NOIM000000000](#), and [NPJX000000000](#).

RESULTS

National surveillance. A total of 40,205 cases of IPD were reported from 2005 through 2014, of which 70% (28,213/40,205) had viable isolates. Serotypes 1 ($n = 3,340$), 19A ($n = 2,541$), 6A ($n = 2,030$), 14 ($n = 1,898$), 23F ($n = 1,891$), 6B ($n = 1,681$), 4 ($n = 1,494$), 19F ($n = 1,470$), 12F ($n = 1,387$), and 8 ($n = 1,109$) were the most common serotypes identified. IPD coinfection with more than one serotype was identified in 35 (0.1%) patients with available viable isolates. In two of the patients, infections were caused by isogenic strains and have been described elsewhere (12). In the present study, we describe the remaining 33 cases and corresponding isolates.

Characteristics of patients and isolates causing dual-serotype IPD. In 26 of the 33 patients with dual serotypes, both isolates were obtained from the same specimen (Table 1). For the remaining seven patients, different serotypes were identified from two different specimens (Table 1). For the 26 patients with dual serotypes, 25 were identified during routine serotyping, and 1 case was detected during antimicrobial susceptibility testing. Pairs of isolates were predominantly recovered from blood (21/33, 64%), followed by CSF (11/33, 33%). Serotypes 6B ($n = 10$), 14 ($n = 8$), 6A ($n = 7$), 19A ($n = 5$), 19F ($n = 5$), and 1 ($n = 4$) were the most common serotypes identified. For 30 of the 33 patients (91%) at least one of the detected serotypes in each pair was a PCV13 serotype (serotype 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F). For 21 (62%) patients both isolates were PCV13 serotypes, 9 (32%) patients had infection with one PCV13 and one non-PCV13 serotype, and for 3 (9%) patients both isolates were non-PCV13 serotypes. Serotype 6B/14 ($n = 3$), 6A/14 ($n = 2$) and 6A/19F ($n = 2$) combinations were detected more than once, whereas all other cases ($n = 27$) had

TABLE 1 Characteristics of patients and isolates causing invasive pneumococcal dual-serotype disease, South Africa, 2005 to 2014^a

Patient	Age (yr)	HIV status	In-hospital outcome	Source specimen(s)	Yr	Isolate 1			Isolate 2		
						Serotype	Antibiogram	ST (CC)	Serotype	Antibiogram	ST (CC)
1	1	Unknown	Unknown	Blood	2005	6A	P, COT	1094 (1094)	14	P, COT	7270
2	1	Negative	Recovered	Blood	2005	6A	P, COT	1094 (1094)	19F	P, COT	347 (347)
3	1	Positive	Recovered	Blood and CSF	2005	14	P, E, CD, T	NV	18C	S	NV
4	29	Positive	Recovered	Blood	2005	14	COT	10237	19A	P	2062 (2062)
5	1	Positive	Died	Blood and CSF	2006	6B	P, COT	7738	19F	P, COT	10536 (347)
6	1	Unknown	Unknown	Blood	2006	15B	P, COT	7052	19F	P, COT	6277
7	4	Unknown	Unknown	Blood	2006	22	COT	5577	23F	COT	36
8	35	Positive	Recovered	Blood	2006	9N	COT	NV	18A	NV	NV
9	85	Unknown	Died	Blood	2007	3	S	NV	35B	P, T, COT	NV
10	5	Unknown	Recovered	Blood	2007	6A	P, E, CD, T, COT	1094 (1094)	14	P, C, T, COT	9901
11	1	Positive	Recovered	Blood	2007	6A	P, COT	1094 (1094)	19F	P, COT	347 (347)
12	3	Unknown	Died	Blood and CSF	2007	6A	S	NV	23F	P, E, CD, C, COT	NV
13	50	Negative	Recovered	Blood	2007	8	S	NV	23F	P, E, T, COT	NV
14	34	Unknown	Unknown	CSF	2008	3	S	458 (458)	19A	P, E, COT	2062 (2062)
15	28	Unknown	Unknown	Blood	2008	6B	P, T, COT	10537	14	S	10239
16	0	Positive	Died	CSF	2008	6B	COT	138	14	P, T, COT	230
17	0	Unknown	Unknown	CSF	2008	9V	P, COT	3454	15A	S	10065
18	0	Positive	Recovered	Blood and CSF	2008	19A	P, COT	2062 (2062)	22	S	445 (445)
19	0	Negative	Died	CSF	2009	1	S	NV	22A	COT	NV
20	1	Unknown	Died	Blood	2009	6A	P, E, COT	NV	16F	S	NV
21	32	Positive	Died	Blood	2009	6B	P, E, T, COT	6285	14	COT	2914
22	12	Positive	Recovered	Blood	2009	6B	P, COT	10066	19F	P, COT	8326
23	0	Negative	Recovered	Blood	2009	9V	S	1492	14	P, E, CD, T, COT	8782
24	30	Unknown	Unknown	Blood	2010	1	S	NV	18C	P, COT	NV
25	0	Positive	Recovered	CSF	2010	6A	P, COT	1094 (1094)	12F	T, C, COT	989
26	0	Unknown	Unknown	CSF	2010	6B	P, COT	NV	18C	S	NV
27	37	Unknown	Unknown	Blood	2010	6B	P, COT	185 (185)	19A	P, COT	6261
28	Unknown	Unknown	Unknown	BPS	2010	22F	S	ST445 (445)	33F	S	10068
29	9	Unknown	Unknown	Blood and CSF	2011	1	S	NV	6B	P, E, T, COT	NV
30	40	Negative	Died	Blood	2011	1	S	9067 (217)	19A	P, COT	2062 (2062)
31	20	Unknown	Died	Blood and blood	2011	3	S	NV	6B	P, E, CD, T	NV
32	49	Unknown	Unknown	Blood and pleural fluid	2011	6B	P, COT	NV	23F	P, E, T, COT	NV
33	1	Negative	Died	Blood	2012	16F	COT	NV	34	S	NV

^aST, sequence type; CC, clonal complex; CSF, cerebrospinal fluid; BPS, brain pus swab; NT, nontypeable; C, chloramphenicol; CD, clindamycin; COT, co-trimoxazole; E, erythromycin; P, penicillin; T, tetracycline; S, susceptible to all tested antibiotics; NV, nonviable (for 13 cases isolates were nonviable on subculture for both isolates or one of the codetected isolates).

unique serotype pairs (Table 1). For the majority (24/33, 73%) of patients, the coinfecting isolates had different antibiotic susceptibility profiles (Table 1). For the remaining nine patients, the pairs had identical antibiograms, including a patient where the pair was susceptible to all tested antimicrobial agents, one patient where both isolates were nonsusceptible to co-trimoxazole only and seven patients where the pairs of isolates were nonsusceptible to penicillin and co-trimoxazole.

Factors associated with dual-serotype IPD infection. IPD patients from enhanced sites, where additional clinical data were collected, represented 41% (11,701/28,213) of patients with viable isolates. Dual serotypes were identified in 0.2% (21/11,701) of patients from enhanced sites (Table 2). On multivariable analysis, a dual serotype was associated with patients less than 5 years of age (adjusted odds ratio [aOR], 4.7; 95% confidence interval [95% CI], 1.8 to 11.7), underlying illness other than HIV (aOR, 2.8; 95% CI, 1.1 to 6.6), and death (aOR, 2.5; 95% CI, 1.08 to 6.09) (Table 2).

Genotypic characteristics of dual-serotype IPD isolates. Paired isolates were available for WGS in 20 of the 33 cases ($n = 40$ isolates). For 13 patients, one isolate ($n = 10$) or both isolates ($n = 3$) were no longer viable on subculture from long-term storage. The genome coverage for the isolates ranged from $31\times$ to $607\times$.

Among each pair of isolates from the same patient none were identical by MLST (Table 1). No predominant genotype was identified among serotype 14 isolates ($n = 7$ unrelated STs); however, clonal complex 1094 (CC1094), CC185, CC2062, and CC347 were common among the 6A (5/5), 6B (2/6), 19A (3/5), and 19F (4/6) isolates, respectively. Core genomes of all 40 isolates clustered with corresponding isolates of the same serotype and sequence type (see Fig. S1 in the supplemental material).

Among the 40 dual-serotype isolates, 1,794 accessory genes were identified (see Table S2 in the supplemental material). A total of 701 (39%) of the 1,794 genes coded for known proteins, and the remaining 1,093 coded for hypothetical proteins. *queT* and *infC* (coding for queuosine precursor transporter QueT and translation initiation factor IF-3, respectively) were the only accessory genes identified among all coinfecting serotypes and were both found in 39 of the 40 isolates (and not found in 59 isolates from IPD patients with a single serotype) (see Table S2 in the supplemental material). Beside *queT* and *infC*, only two other genes (*ald-2* and *serB*) were identified among pairs of isolates from the same patient. *ald-2* shared by the 6A and 12F pair (patient 25 in Table 1) was also identified among other 6A isolates ($n = 4$) from IPD patients with dual serotypes. *serB* was shared by a serotype 22F/33F pair from the same patient (patient 28 in Table 1). CHY zinc finger, streptococcal histidine triad protein, and DNA alkylation repair enzyme (each encoded by more than one accessory gene) were common proteins, distributed among at least 10 serotypes.

For the 40 isolates that were sequenced, 10 ($n = 19$ isolates), 12 ($n = 20$ isolates), and 10 ($n = 22$ isolates) previously identified TPD amino acid sequences (alleles) of PBP1a, PBP2b, and PBP2x were identified, respectively. We also identified new sequences that did not match any of the sequences in the CDC databases ($n = 15$, 14, and 12 isolates for the TPD amino acid sequences of the three PBPs, respectively). For the remaining six isolates, PBP sequences could not be identified. Twenty-three PBP types (PBP1a, PBP2b, and PBP2x allelic profiles) were identified for the 33 isolates with PBP sequences. None of the pairs of isolates from the same patient shared the same PBP type.

DISCUSSION

We describe cases of invasive pneumococcal disease caused simultaneously by two different strains. This phenomenon has been reported by others mainly in immunocompromised patients or in young children (5–7). Similarly, in our study, children younger than 5 years and patients with underlying illness other than HIV were more likely to have dual-serotype infection compared to older individuals and those without underlying illness, respectively. The lack of association between HIV infection and dual infection is possibly due to the low number of dual-serotype patients with known HIV status. We identified increased odds of death among patients with dual-serotype

TABLE 2 Univariate and multivariable analysis of factors associated with dual-serotype infection among patients with invasive pneumococcal disease at enhanced sites, South Africa, 2005 to 2014

Characteristic	No. (%) of cases			Univariate analysis		Multivariable analysis	
	Total	Cases with single serotype	Cases with dual serotypes	OR (95% CI)	<i>P</i> ^a	aOR (95% CI)	<i>P</i> ^a
Yr	11,701	11,680	21				
2005	1,583 (14)	1,580 (14)	3 (14)	Reference			
2006	1,521 (13)	1,519 (13)	2 (10)	0.6 (0.1–4.1)	0.6		
2007	1,416 (12)	1,411 (12)	5 (24)	1.86 (0.4–7.8)	0.3		
2008	1,418 (12)	1,416 (12)	2 (10)	0.7 (0.7–0.1)	0.7		
2009	1,458 (12)	1,453 (12)	5 (24)	1.8 (0.4–7.5)	0.4		
2010	1,173 (10)	1,172 (10)	1 (5)	0.4 (0.04–4.3)	0.4		
2011	997 (9)	995 (9)	2 (10)	1.05 (0.1–6.3)	0.9		
2012	804 (7)	803 (7)	1 (5)	0.6 (0.06–6.3)	0.7		
2013	732 (6)	732 (6)	0				
2014	599 (5)	599 (5)	0				
Gender	11,690	11,669	21				
Male	5,760 (49)	5,746 (49)	14 (67)	Reference			
Female	5,930 (51)	5,923 (51)	7 (33)	0.5 (0.1–1.2)	0.1		
Age group	11,691	11,670	21				
≥5 yr	7,976 (68)	7,969 (68)	7 (33)	Reference		Reference	
<5 yr	3,715 (32)	3,701 (32)	14 (67)	4.3 (1.7–10.6)	0.002	4.7 (1.8–11.7)	0.001
HIV status	9,006	8,990	16				
Negative	2,472 (27)	2,466 (27)	6 (38)	Reference			
Positive	6,534 (73)	6,524 (73)	10 (63)	0.6 (0.2–1.7)	0.3		
Underlying illness other than HIV ^b	11,701	11,680	21				
No	7,942 (68)	7,966 (68)	9 (43)	Reference		Reference	
Yes	3,759 (32)	3,747 (32)	12 (57)	2.8 (1.1–6.7)	0.01	2.8 (1.1–6.6)	0.01
Outcome	11,470	11,449	21				
Recovered	8,115 (71)	8,104 (70)	11 (52)	Reference		Reference	
Died	3,355 (29)	3,345 (29)	10 (48)	2.2 (0.9–5.1)	0.07	2.5 (1.08–6.09)	0.03
Specimen type	11,701	11,680	21				
Cerebrospinal fluid	3,384 (29)	3,376 (29)	8 (38)	Reference			
Blood	7,645 (65)	7,632 (65)	13 (62)	0.7 (0.2–1.7)	0.46		
Other	672 (5)	672 (6)	0				
Serotype ^c	11,701	11,680	21				
Non-PCV13	3,585 (31)	3,583 (31)	2 (10)	Reference			
PCV13	8,116 (69)	8,097 (69)	19 (90)	4.2 (0.9–18.05)	0.05		
Penicillin susceptibility ^d	11,701	11,680	21				
Susceptible	7,333 (63)	7,326 (63)	7 (33)	Reference			
Non-susceptible	4,368 (37)	4,354 (37)	14 (67)	3.3 (1.3–8.3)	0.009		
Multidrug resistance ^d	11,701	11,680	21				
No	9,501 (81)	9,487 (81)	14 (67)	Reference			
Yes	2,200 (19)	2,193 (19)	7 (33)	2.1 (0.8–5.3)	0.09		

^aSignificant *P* values (*P* < 0.05) are indicated in boldface type. OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval.

^bUnderlying illness includes asthma, chronic lung disease, cirrhosis/liver failure, chronic renal failure, heart failure, heart failure, valvular heart disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephrotic syndrome, spinal cord injury, seizure disorder, and emphysema or cancer.

^cFor individuals with dual serotypes, the pair was classified as PCV13 if at least one of the isolates was a PCV13 serotype (serotype 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F) or non-PCV13 if both isolates were serotypes other than PCV13.

^dFor individuals with dual serotypes, the pair was classified as penicillin nonsusceptible and/or multidrug resistant if at least one of the isolates was penicillin nonsusceptible and/or multidrug resistant.

infections compared to those with single infections. The association between infection with dual serotypes and young children is probably a reflection of underdeveloped immune systems and higher carriage rates of the pneumococcus in children, with increased probability of invasive disease by more than one serotype in children carrying multiple serotypes (23).

Our study has some limitations. We were not able to verify dual serotypes from original clinical specimens as primary isolation is carried out at the diagnostic laboratories, following which, isolates are submitted to our laboratory for surveillance purposes. However, given the rarity of dual serotypes in IPD, such cases were investigated carefully, and several measures were taken to rule out laboratory error. The detection of multiple serotypes from the same clinical specimen has been shown to be improved by detecting directly from specimens rather than from culture plates (24). As such, we may have missed some cases because we did not serotype directly from specimens. In addition, serotypes (particularly those present in low copy numbers in original specimens) may have been missed because more sensitive methods such as molecular techniques were not used for serotyping.

Our laboratory has been working with large numbers of pneumococcal isolates for more than two decades. Given the challenges of Quellung in detecting multiple serotypes from one source, we believe that the high level of experience and expertise of our technicians made discoveries of dual serotypes during routine serotyping possible. We also believe that the use of molecular serotyping could improve our detection rate.

The most common serotypes identified among patients with dual infection were PCV13 serotypes 1, 6A, 6B, 14, 19A, and 19F. This is in line with the overall IPD serotype prevalence prior to routine use of PCV13 in South Africa (25–27) during the period in which the majority of these cases occurred, where more than 80% of IPD cases were due to PCV13 serotypes (27). It is interesting, however, that the majority of coinfecting serotypes (6A, 6B, and 19F) identified in our study are known to have low invasive disease potential (28). The significant association between dual infection and underlying illness could explain the vulnerability of these patients to invasive disease with more than one strain.

Similar to dual-serotype IPD cases reported previously where coinfecting isolates from the same patient were genotypically unrelated (5, 6), the majority of our isolate pairs were different strains as indicated by unrelated sequence types. The core genome of each isolate clustered with genomes of other South African IPD isolates of the same serotype and sequence type. Genotypes identified were common genotypes among those serotypes circulating in South Africa prior to routine use of PCV (29). Our study is limited to the detection of dual-serotype infections and does not include dual infections by the same serotype but differing genotype.

A study in The Gambia (4), with a high prevalence (19%) of multiple-serotype carriage, reported evidence of recombination in 34% (49/144) of single serotype and cocarried pneumococcal isolates (23, 30). Two accessory genes identified in all genomes (*queT* and *infC*) and two from genomes of two individual pairs (*ald-2* and *serB*) were the only accessory genes common between pairs of isolates from the same IPD cases. The lack of sequence similarity between the majority of accessory genes and unique PBP types between pairs is evidence that during each episode of invasive disease in this study, there was little or no genetic exchange between coinfecting pairs of isolates. For these patients, the isolates could have invaded too soon after acquisition, giving limited time for cocarriage and genetic exchange.

Pneumococcal conjugate vaccines (PCV7, PCV10, and PCV13) prevent acquisition and disease of the most prevalent disease-causing pneumococcal serotypes (27, 31, 32). We did not detect any case of dual-serotype infection in the last 2 years of the study, nor in 2015 to 2016 (data not shown), most likely reflecting the decline in pneumococcal carriage and IPD in South Africa after routine PCV use (27).

Our findings suggest that IPD due to dual serotypes is rare. Nevertheless, it is important to monitor such infections since they are more likely to occur in children, probably because of their immature immunity, and in patients with underlying illness because of impaired immune response. In addition, compared to patients with a single serotype, these patients were more likely to die, probably because the presence of two serotypes exacerbated the disease. This study highlights the need for the routine use

of more sensitive methods in laboratories to increase their ability to detect IPD cases with more than one serotype.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.01149-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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